University of Aveiro Chemistry Department
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Bases genéticas da Eritrocitose Congénita- Pesquisa de novas mutações e genes associados e atualização das bases de dados online

Genetic Basis of Congenital Erythrocytosis - Search for new mutations and associated genes and update of online databases

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“Everything happens for a reason,

Even if you not know the reason now,

You will soon enough,

Just give it some time”

Unknown

Jury

Presidente — Professora Doutora Rita Maria Pinho Ferreira
Professora Auxiliar, Universidade de Aveiro

Doutor Licínio Manuel Mendes Manco
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Palavras-Chave

Eritrocitose Congénita, eritropoietina, recetor da eritropoietina, via de hipóxia, Hemoglobinas de alta afinidade.

Resumo

Eritrocitose Congénita (EC) designa um grupo de patologias que podem ser primárias ou secundárias, sendo classificadas com base nos níveis de eritropoietina (EPO). A EC primária, devida a alterações nos percursores eritroides, é causada por alterações no recetor da eritropoietina (EpoR) devido a mutações nos genes *EPOR* e *SH2B3*. O EpoR fica permanentemente ativado, o que leva à diminuição dos níveis de EPO. A EC secundária pode ser causada por alterações de componentes na via de sensibilização ao oxigénio, devido a mutações nos genes *VHL*, *EGLN1* e *EPAS1*, ou devido a hemoglobinas de alta afinidade para o oxigénio em consequência de mutações nos genes *HBA*, *HBB* e *BPGM*. Mutações nestes genes promovem um aumento dos níveis de EPO. Apesar de já estarem descritas causas moleculares para a origem da EC, cerca de 70% dos doentes ainda permanecem sem uma causa genética identificada.

Neste estudo foram analisadas 125 amostras de indivíduos com suspeita de EC, seguidos no Centro Hospitalar e Universitário de Coimbra ou enviados de outros hospitais portugueses/internacionais. Os testes laboratoriais foram orientados com base na história clínica e familiar e nos níveis de EPO, e incluíram a sequenciação dos genes: *EPOR*, *SH2B3*, *VHL*, *EGLN1*, *EPAS1*, *HBA*, *HBB*, *BPGM*.

Foram identificadas 5 mutações: 3 no gene *VHL* (c.74C>T, p.Pro25Leu; c.154G>T, p.Glu52*; c.241C>T, p.Pro81Ser), 1 no gene *EGLN1* (c.1216G>C, p.Gly406Arg) e 1 no gene *HBB* (Hb San Diego [HBB: c.328G>A, p.Val110Met]). Mutações no gene *VHL* têm uma transmissão autossómica recessiva estando descritos casos esporádicos em que se encontra apenas uma mutação, também neste estudo todas as mutações encontradas estão em heterozigotia. O mecanismo subjacente nestes casos ainda permanece por elucidar. A mutação encontrada no gene *EGLN1* não está descrita na literatura, segundo a análise in sílico é uma variante patogénica, pelo que deverá ser a causa da EC. No gene *HBB* a variante foi identificada num individuo de origem turca, sendo a primeira descrição desta variante na Turquia.

Concluindo, neste estudo foi possível identificar a causa molecular da EC em 5/125 doentes estudados. Foram encontradas 4 mutações descritas e 1 nova mutação. No entanto, 120 doentes ainda permanecem sem diagnóstico molecular. Isto demonstra que mais estudos são necessários para entender esta doença. O uso da tecnologia de *Next Generation Sequencing* (NGS) pode ser uma ferramenta valiosa no estudo da EC, uma vez que pode identificar outros genes que possam estar envolvidos na EC.

Keywords

Congenital Erythrocytosis, erythropoietin, erythropoietin receptor, oxygen-sensing pathway, high oxygen affinity haemoglobin

Abstract

Congenital Erythrocytosis (CE) belongs to a diseases group that can be primary or secondary, and are classified based on EPO levels. Primary CE, due to mutations in the erythroid precursors, is caused by changes in EPO receptor (EpoR) due to mutations in *EPOR* and *SH2B3* genes. EpoR is permanently activated, which leads to a decrease in EPO levels. Secondary CE can be caused by defects in the components of the oxygen-sensing pathway, namely mutations in *VHL*, *EGLN1* and *EPAS1*, or due to congenital defects such as Hbs with increased oxygen affinity, due to mutations in the *HBB*, *HBA* or *BPGM*. Mutations in these genes leads to an increase of EPO levels. Despite important discoveries in the molecular pathogenesis of CE, in about 70% of the patients the genetic causes remain to be identified.

In this study were analysed 125 samples of individuals with suspicious of CE, followed in Centro Hospitalar e Universitário de Coimbra or from other Portuguese/international hospitals. Laboratory testing was guided by clinical and familiarhistory and EPO levels, and included: *EPOR*, *SH2B3*, *VHL*, *EGLN1*, *EPAS1*, *HBA*, *HBB*, *BPGM* sequencing.

Were identified 5 mutations: 3 in *VHL* gene (c.74C>T, p.Pro25Leu; c.154G>T, p.Glu52*; c.241C>T, p.Pro81Ser), 1 in *EGLN1* gene (c.1216G>C, p.Gly406Arg) and 1 in *HBB* gene (Hb San Diego [HBB: c.328G>A, p.Val110Met]). Mutations in *VHL* gene have an autosomal recessive inheritance, with sporadic cases in which only one mutation is found, also in this study all the mutations found are in heterozygosity. The underlying mechanism in these cases still remains to be elucidated. The mutation found in the *EGLN1* gene is not described in the literature, according to in silico analysis it is a pathogenic variant and should therefore be the cause of CE. In the *HBB* gene the variant was identified in an individual of Turkish origin, the first description of this variant in Turkey

In conclusion, in this study was possible to identify the CE molecular aetiology in 5/125 patients studied. It was found 4 described mutations and 1 new mutation. However, 120 patients still remain without molecular diagnosis. This demonstrates that more studies are needed to understand this disease. The use of Next Generation Sequencing (NGS) technology can be a valuable tool in the study of CE as it can identify other genes that may be involved in CE.

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List of Abbreviations

1,3 BPG- 1,3 bisphosphoglycerate

2,3-BPG- 2,3 bisphosphoglycerate

BPGM- Biphosphoglycerate mutase

CE- Congenital Erythrocytosis

CISH- Cytokine-inducible SH2 domain containing protein 2

CO- Carbon monoxide

CO^2- Carbon dioxide

DNA- Deoxyribonucleic acid

ECYT1- CE type 1

ECYT2- CE type 2

ECYT3- CE type 3

ECYT4- CE type 4

EPO- Erythropoietin

EpoR- Erythropoietin receptor

Grb2- Growth factor receptor protein 2

Hb- Haemoglobin

Hbs- Haemoglobins

Hct- haematocrit

HIF2α – Hypoxia-inducible factor 2α

HPLC- High-performance Liquid Chromatography

JAK2- Janus kinase 2

LNK- Lymphocyte Adaptor Protein

MAPK- Mitogen-activated protein kinase

NGS- Next Sequencing Generation

O_2- Oxygen

PCR- Polymerase Chain Reaction

PH domain- Pleckstrin homology domain

PHDs- Propyl hydroxylases

PHD2- Propyl hydroxylase domain-containing protein 2

PI3K- Phosphatidylinositol 3-kinase

RBC- Red blood cells

SH2 domain- Src homology 2 domain

SHC-CHUC- Serviço de Hematologia Clínica do Centro Hospitalar e Universitário de Coimbra

SOCS- Suppressor of cytokine signalling

STAT5- Signal transducer activator of transcription 5

VHL- von Hippel Lindau protein

WGS- Whole Genome Sequencing

Introduction

1. Erythrocytosis

1.1 General Overview

The supply of oxygen (O_2) to the tissues is accomplished by red blood cells (RBC) containing haemoglobin (Hb). Hb is an iron-containing molecule in the blood that is responsible to transports oxygen to tissues. The membrane of RBC is rich in proteins and lipids, which confer essential characteristics for physiological function such as deformability and stability (1). Due to the lack of the nucleus in the mature RBC, the storage capacity of Hb is increased. The production of these cells is maintained through mechanisms of homeostasis, such as vasoconstriction or coagulation (1,2). However, any imbalance in these mechanisms may lead to an increase in the production of RBC, causing pathologies namely as erythrocytosis (1,3).

Erythrocytosis is a rare disease characterized by a high haematocrit (Hct), increased levels of Hb and an increase in the number of RBC when compared with the reference values for normality (3,4,5). These values vary according to gender, age and living altitude. Erythrocytosis is diagnosed when values measured in adults are as follows: Hb> 18.5 g/dL or Hct> 52% in men and Hb> 16.5g/dL or Hct> 48% in women (6). Due to an increase in the number of RBC, this disorder leads to the increase blood viscosity which may lead to the emergence of symptoms, such as headaches, dizziness and/or pruritus after bathing. The most common strategy applied to alleviate these symptoms has been to perform regular phlebotomies. This technique consists of the periodic collection (usually every 3 months) of a certain volume of blood (about 500 mL), with the purpose of decreasing the Hct (2).

1.2 Classification of Erythrocytosis

Erythrocytosis is first classified based on levels of erythropoietin (EPO), the hormone that regulates the production of RBC. EPO is produced primarily in the kidney, and in lesser amounts in the liver, and circulates freely in the blood (7,8,9). Hypoxia (blood oxygen deficiency) is one of the main stimuli for EPO production. On suspicion of erythrocytosis, low levels of EPO are associated with primary erythrocytosis, while normal to high levels are associated with secondary erythrocytosis. However, individuals without erythrocytosis can also show low/high levels of EPO (8).

Primary erythrocytosis occurs due to intrinsic defects in erythroid precursors, namely proerytroblasts and erythroblasts, among others, which reflect the low levels of EPO and the hypersensitivity of erythroid precursors to EPO (5). On the other hand, secondary erythrocytosis

manifests through increased production of EPO for unknown reason, or due to tissue response to hypoxia. This increment in EPO production occurs through causes extrinsic to the erythroid precursors, such as hypoxia, thus promoting a higher production of RBC (5,10).

In addition to evaluating EPO levels, it is important to unravel the underlying cause of erythrocytosis, which can be congenital or acquired (3). The latter is the most frequent forms of erythrocytosis and should be first excluded. This also occurs in renal, cardiac, hepatic and pulmonary diseases, leading to an increase of EPO, and also due to hepatoma, haemagioblastoma, nephroblastoma, endocrine and uterine tumours, and polycythaemia Vera, which may cause autonomic synthesis of EPO (3,15,16,17). All of the aforementioned conditions are associated with normal to high levels of EPO, except for polycythaemia Vera, which is associated with low levels of EPO (11,17,18). Polycythaemia Vera is a clonal form of primary erythrocytosis and its usually associated with Janus kinase (JAK2) mutations (JAK2 V617F or exon 12) (19,20). Finally, people living at high altitudes also show a high number of RBC, due an increase of EPO, which is classified as an acquired cause since it is triggered due to external factors (13).

Congenital erythrocytosis (CE) is an inherited disease that is present since birth. Germline mutations result in the translation of defective proteins, either the regulation of EPO and its receptor (EpoR) or in the oxygen sensing pathway, both leading to an increase of RBC. Additionally, CE may be either primary or secondary depending on the mutated gene, and as a consequence of these mutations, EPO levels are affected (3,5,12). Primary CE is associated with low levels of EPO and hypersensitivity of erythroid precursors to EPO, and is only found in 12% of the patients with an identified molecular cause (14). The usual known form is caused by mutations in the *EPOR* gene, and more recently mutations in *SH2B3* gene, which encodes the lymphocyte adaptor protein (LNK), were described (14,22,23,24).

On the other hand, secondary CE is associated with an increase of EPO levels which may arise due to defects in the components of the oxygen sensing pathway, namely mutations in prolyl hydroxylase domain-containing protein 2 (PHD2, encoded by *EGLN1* gene), hypoxia-inducible factor 2α (HIF2α, encoded by *EPAS1* gene) and von Hippel Lindau protein (VHL, encoded by *VHL* gene) (13,16,22,25). Additionally, secondary CE can be related to Hb variants with high oxygen affinity, due to mutations in the beta (β) or alpha (α) globin genes (*HBB*, *HBA*) or due to a 2,3 bisphosphoglycerate (2,3-BPG) deficiency, that result from mutations in the *BPGM* gene. High oxygen affinity Hbs causes tissue hypoxia, and promotes a higher production of RBC (8,26,27,28).

Finally, when the cause of erythrocytosis is still elusive after extensive testing, it is classified as Idiopathic Erythrocytosis. This corresponds to the majority of cases suspected of having erythrocytosis (17). Table 1 summarizes causing mutations of erythrocytosis.

Table 1- Classification of Erythrocytosis and causing mutations.

<table>
<tr><th colspan="2">Primary (↓levels of EPO)</th><th colspan="2">Secondary (↑ levels of EPO)</th></tr>
<tr><th>Congenital</th><th>Acquired</th><th>Congenital</th><th>Acquired</th></tr>
<tr><td rowspan="4">Congenital Erythrocytosis type 1 (ECYT1)- due to mutations in EPOR and SH2B3</td><td rowspan="4">Polycythaemia Vera (PV) – due to JAK2V617F and JAK2 exon12 mutations</td><td>Hb with high affinity to O_2 – mutations in HBB and HBA and deficiency in 2,3-BPG- mutations in BPGM</td><td rowspan="4">Increase of EPO due to:
-Cardiac, pulmonary, liver and kidney diseases
Autonomous EPO synthesis caused by:
- nephroblastoma
- hepatoma
- haemagioblastoma
- Endocrine tumour
- Uterus tumour</td></tr>
<tr><td>Congenital Erythrocytosis type 2 (ECYT2)- mutations in VHL</td></tr>
<tr><td>Congenital Erythrocytosis type 3 (ECYT3)- Mutations in PHD2 (EGLN1)</td></tr>
<tr><td>Congenital Erythrocytosis type 4 (ECYT4)- Mutations in HIF2α (EPAS1)</td></tr>
<tr><td colspan="4">Idiopathic- unknown molecular cause</td></tr>
</table>

2. Congenital Erythrocytosis- Molecular Mechanisms

Nowadays there are 8 genes described as associated with CE, which are the following: *EPOR*, *SH2B3*, *VHL*, *EGLN1*, *EPAS1*, *BPGM*, *HBB* e *HBA* (13). Low levels of EPO are indicative of defects in EPO signalling pathway, unlike the normal to high levels, which are indicative of defects in the oxygen sensing pathway or are related to Hb variants with high oxygen affinity. The various genes involved in this pathology are associated with the different types of CE and different types of inheritances (12,13,27,30). These characteristics are shown in table 2.

Table 2- Congenital Erythrocytosis- Disease group, gene, location, number of exons, inheritance, and proteins related to genes involved in Congenital Erythrocytosis (5).

DISEASE GROUP (OMIM NUMBER)	GENE (OMIM NUMBER)	LOCATION	NUMBER OF EXONS	INHERITANCE	PROTEIN
ECYT1 (133100)	*EPOR* (133171)	19p13.2	8	Dominant (*de novo* cases described)	EPO receptor (EpoR)
	SH2B3 (605093)	12q24.12	8	-	Lymphocyte adaptor protein- LNK
ECYT2 (263400)	*VHL* (608537)	3p25.3	3	Recessive (dominant cases described)	von Hippel Lindau protein - VHL
ECYT3 (609820)	*EGLN1* (606425)	1q42.1	5	Dominant	Prolyl hydroxylase domain-containing protein 2- PHD2
ECYT4 (611783)	*EPAS1* (603349)	2p21	16	Dominant	Hypoxia Inducible Factor 2α - HIF-2α
HIGH OXYGEN AFFINITY: HBA(-), HBB (-) BPGM (222800)	*HBA* (*HBA1/HBA2*) (141800), *HBB* (141900)	11p15.4 16p13.3	3 3	Dominant	Haemoglobin- Hb
	BPGM (613896)	7q33	2	Recessive	Biphophoglycerate mutase- BPGM

2.1 Primary Congenital Erythrocytosis

2.1.1 ECYT1- EpoR (*EPOR* gene) and LNK (*SH2B3* gene)

Primary CE is usually characterized by low levels of EPO, hypersensitivity of erythroid precursors to EPO and normal oxygen affinity of Hb. Typically, it occurs due to an autosomal dominant inheritance, arising from mutations in the *EPOR* gene that result in a protein constitutively active, or from mutations in LNK, which is a negative regulator of the cytokine signalling pathway (14). EPO is a cytokine that binds to EpoR which is a homodimer present on the cell surface of erythroid cells. The binding of EPO to EpoR initiates a phosphorylation cascade signalling pathway involved in proliferation, differentiation and prevention of apoptosis (13). The most common mutations in *EPOR* gene are nonsense and are located in exon 8, which is responsible

for encoding the C-terminal negative regulatory domain of the protein. Mutations in this exon results in a truncated protein (14,18,19).

As shown in figure 1, upon binding of EPO to EpoR, homodimerization of EpoR occurs and leads to autophosphorylation of the associated tyrosine JAK2. Activation of JAK2 protein mediates a signalling cascade that lead to the phosphorylation of tyrosine residues in the cytoplasmic domain of the EpoR, which serves as a docking site for the signal transducer and as an activator of transcription 5 (STAT5) protein, the adaptor molecule growth factor receptor protein 2 (Grb2) and phosphatidylinositol 3-kinase (PI3K). Activated STAT5 homodimerizes and is translocated to the nucleus where it binds to the promoter of target genes, and thus *EPOR* gene transcription is initiated. Cell proliferation is supported by the activation of Ras/mitogen associated protein (Ras/MAP) kinase through Grb2. In addition, activation of PI3K results in the phosphorylation of Akt and the induction of several anti-apoptotic proteins, such as Bcl-2, Bclx and protein kinase B, thus prolonging cell survival increasing proliferation and inhibiting apoptosis (20).

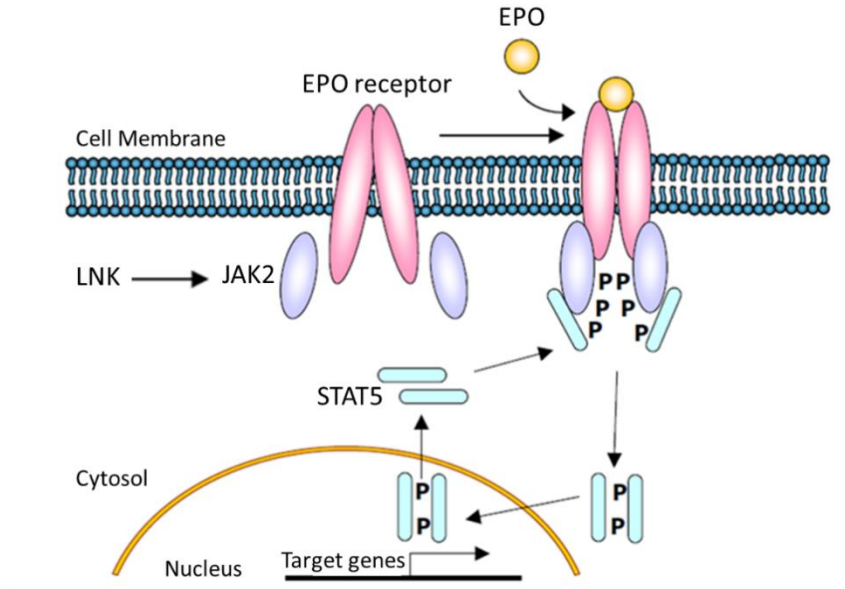

Figure 1- Representation of the EPO signalling and JAK/STAT pathway.

EPO binds to its receptor (EpoR) and autophosphorylates, JAK2 dimerises STAT5 that is translocate into the nucleus where it binds to the promoter of target genes. After the binding of EPO to EpoR all signalling pathways are disrupted by the attenuation of JAK2 activation. LNK through its SH2 domain negatively modulates EpoR signalling by attenuating JAK2 activation and regulates EPO-mediated erythropoiesis. Adapted from (13,21,22).

Several different mechanisms are activated to control the EPO responsiveness of erythroid cells so they can tightly regulate and thus prevent uncontrolled erythropoiesis. As a result, around 30 minutes after EPO binding all signalling pathways are disrupted by the attenuation of JAK2 activation (21). Inhibitory proteins are recruited to the receptor, such as the phosphatase SHP-1, which is responsible for the dephosphorylation of EpoR and JAK2 (21). Furthermore, STAT5 induces the transcription of several members of the suppressors of cytokine signalling (SOCS) proteins and, in particular, cytokine-inducible SH2-domain-containing protein (CISH) and SOCS3. CISH is able to bind phosphorylated tyrosines residues on the EpoR thus blocking the association of STAT5 to the receptor. Finally, the role of SOCS3 is to disrupt signalling by targeting the EpoR for ubiquitin mediated proteasomal degradation (13,20,21,23).

The LNK, a negative regulator of the cytokine signalling, is comprised of a proline-rich N-terminal dimerization domain, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and a conserved C-terminal tyrosine residue. LNK negatively modulates EpoR signalling through its SH2 domain by attenuating JAK2 activation and moreover regulates EPO-mediated erythropoiesis (13,21). In the presence of EPO, LNK becomes phosphorylated and is able to block the three important signalling pathways of JAK/STAT, phosphoinositide-3 kinase (Pi3 kinase/AKT) and mitogen-activated protein kinase (MAPK) (21). Figure 1 shows the representation of the EPO signalling and JAK/STAT pathway.

CE mutations found in *EPOR* gene lead to a premature stop codon and hence result in a truncated receptor that lacks the negative regulatory domain. The most common mutations in *EPOR* gene are nonsense and are usually located in exon 8. As a consequence, there is loss of the docking site in SHP-1 and for that reason, when EPO binds to EpoR, the receptor is switched on, but does not get switched off again (13,21). Thus, there is continuous stimulus that drives to an increase of RBC production (13,20).

On the other hand, primary CE can also be due to missense mutations in *SH2B3* gene, which encodes the LNK protein. Although mutations in *SH2B3* gene are usually described in association with myeloproliferative neoplasms, there are a few cases of primary CE associated with mutations in this gene (13,18). As mentioned before, LNK is a negative regulator of the cytokine signalling by attenuating JAK2 activation and consequently EPO mediated erythropoiesis. Mutations in *SH2B3* gene results in a defective LNK protein that does not act as a negative regulator of the JAK/STAT pathway downstream of the cytokine attachment to its receptor and thus lead to increased

downstream erythropoiesis and primary erythrocytosis (with an associated low EPO levels) (13,18,24).

2.2 Secondary Congenital Erythrocytosis

2.2.1 Oxygen sensing pathway- Molecular Mechanisms

Oxygen sensing is a fundamental and essential biological process for the survival of all organisms. Under hypoxia conditions, the oxygen sensing pathway is activated and involve three important proteins- VHL, PHD2 and HIF2α- which are responsible for the regulation of *EPO* gene. In normal conditions (normoxia) oxygen activates propyl hydroxylases (PHDs) which catalyse the hydroxylation of HIF, comprised of an alpha and a beta subunit. PHDs hydroxylated propyl residues located within the HIFα allow the binding of HIFα to the VHL protein. This binding induces ubiquitination, which targets them for proteasomal degradation, in normoxia conditions. However, a reduction of the oxygen levels leads to a hypoxia state under which HIFα is stabilized due to the decrease of its hydroxylation. This leads to translocation of HIFα into the nucleus, where it binds to the subunit HIFβ, both forming an active heterodimer, which then binds to hypoxia responsive elements within the genome. This event leads to the activation of target genes and increased production of proteins such as EPO (figure 2).

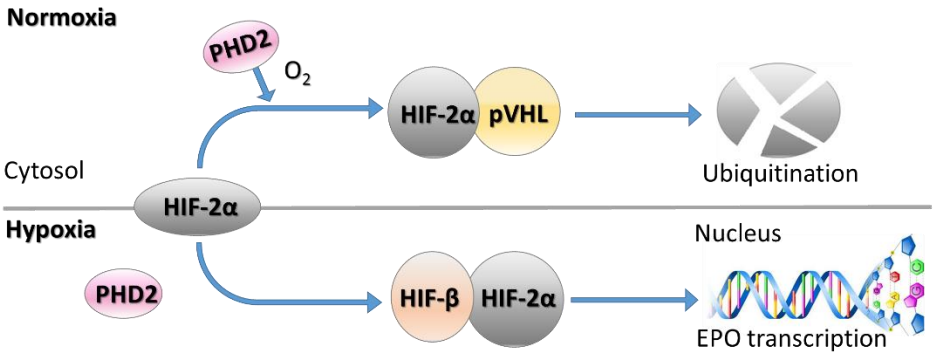

Figure 2- Oxygen-sensing pathway.

In normoxia condition the alpha subunit of hypoxia inducible factor 2α (HIF2α) is hydroxylated by propyl hydroxylase domain-containing protein 2 (PHD2) and binds the VHL protein (pVHL). This process induces the ubiquitination of HIF2α in the proteasome. In hypoxia, HIF-2α is stabilized and translocated into the nucleus where binds to the β subunit, which causes the production of a number of target proteins including EPO. Based on (13).

2.2.1.1 ECYT2- VHL (*VHL* gene)

VHL gene has two translational initiation sites- +1 and +54- that encode two proteins, pVHL30 (larger) and pVHL19 (shorter), respectively. Both are functional proteins and allow the binding of HIFα (25). The first mutation described in the oxygen sensing pathway was in the *VHL* gene. This mutation was identified in a large cohort of individuals with erythrocytosis in the Chuvash region of Russia. Chuvash polycythaemia is an endemic disorder, characterized by normal or elevated EPO levels and inheritance is autosomal recessive and due to homozigoty for c.598C>T, p.Arg200Trp (R200W) mutation in *VHL* gene that results in a lost-of function mutation (13,15,26,27). It was shown that the mutant protein had reduced activity as a negative regulator of HIF gene transcription, resulting in increased expression of HIF, and thus target genes including EPO (27). Later, this mutation was identified in other countries, such as England, Pakistan and most recently in the Italian island of Ischia (28,29).

CE due to mutations in *VHL* gene is a recessive disease, but the occurrence of individuals heterozygous for *VHL* mutations with CE has been described. For example, the mutation p.Arg200Trp, firstly found in homozygous state in Chuvash patients and also identified in combination with other VHL mutations (compound heterozygosity), was also described in CE patients only in the heterozygous state in association with CE type 2 (5,30). However, it is not clear in these cases how erythrocytosis results. So, further investigation into the genome must be carried out, with the aim of identifying new mutations or polymorphisms in association with described ones may be causing the symptoms (9). The symptoms of CE type 2 are usually flushing, low blood pressure, stroke and thrombotic events, varicosities and haemangiomas (15).

2.2.1.2 ECYT3- PHD2 (*EGLN1* gene)

There are three PHD isoenzymes (PHD1, PHD2 and PHD3), but PHD2 is the key enzyme that site-specifically modifies HIF2α in an oxygen-dependent manner, in normoxia conditions (5). Loss-of-function mutations in *EGLN1* gene result in a mutant PHD2 and have an autossomal dominant inheritance. So, erythrocytosis occurs due to the decreased binding ability of the PHD2 and subsequent increased activity of HIFα (25,40).

The first mutation described was the heterozygous mutation c.950C>G, which results in a p.Pro317Arg substitution in a highly conserved region of the protein and was described in three individuals from two generations of the same family (two of them were siblings). All had erythrocytosis with normal to elevated EPO levels in contrast to the normal individuals (5,34).

Another family with a PHD2 mutation resulting in an p.Arg371His substitution showed gain of function of the PHD2 activity *in vitro* (35).

2.2.1.3 ECYT4- HIF2α (*EPAS1*)

The transcription factor HIF2α is hydroxylated by the PHD2 and then degraded by VHL protein. The HIFα factor has three isoforms (HIF1α, HIF2α and HIF3α), but HIF2α is the principal transcription factor that induces the EPO expression, under hypoxia conditions (36). In contrast to loss-of-function mutations described for *VHL* and *EGLN1* genes, mutations in *EPAS1* gene lead to a gain-of-function of HIF2α, meaning that the protein is continuously bound to HIFβ, promoting the *EPO* gene transcription. These mutations have an autosomal dominant mode of inheritance (5,36).

A family with erythrocytosis and elevated EPO levels in three generations was found to have a mutation in the oxygen degradation domain of HIF2α resulting in a Gly537Trp substitution. In *vitro* studies showed that the mutation affects hydroxylation of HIF2α by PHD2 and subsequently the recognition of HIF2α by the VHL protein (37). Other HIF2α missense mutations have been described (Pro534Leu, Met535Val, Met535Ile, Met535Thr, Gly537Trp, Gly537Arg, and Phe540Leu) close to the primary site of hydroxylation Pro531 of HIF2α. In general, these patients had dominant erythrocytosis with normal or raised EPO levels and in some cases, a history of thrombosis or pulmonary hypertension (9,36–39).

2.2.2 High oxygen affinity variants of Haemoglobin

Hb is a tetramer comprised of two alpha and two beta globin chains and four heme groups, each one having a porphyrin ring that contains an iron (Fe) atom in the centre and where oxygen binds reversibly (40). There are two protein conformations that present different oxygen affinities: tense (T) and relaxed (R) with low and high oxygen affinity, respectively, and the existing equilibrium between them is readily observable in the oxygen dissociation curve (figure 3) (13,41,42). Oxygen affinity can vary because of several factors, such as blood pH, levels of 2,3-BPG, haemoglobin structure and temperature. An increase of carbon dioxide (CO_2) leads to a decrease of the pH, and then a decrease of the oxygen affinity, allowing the delivery of O_2 to the tissues (40,42).

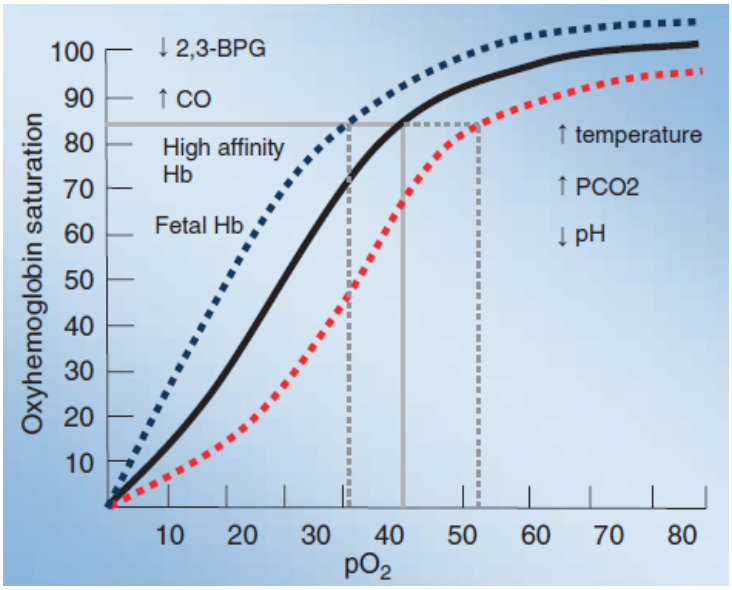

Figure 3- Oxygen dissociation curve.

In red is represented a shift to the right (decreased oxygen affinity) and in blue is represented a shift to the left (high oxygen affinity). Variations of 2,3- biphosphoglycerate mutase (2,3-BPG), CO (carbon monoxide), CO_2 (carbon dioxide), temperature, pH and high oxygen affinity haemoglobins are responsible for the shift of the oxygen dissociation curve (21).

2.2.2.1 *HBA* and *HBB* genes

The α and β chains of Hb are encoded by *HBA* and *HBB* genes respectively. Due to the duplication of the *HBA* gene, there are two genes- *HBA1* and *HBA2* (13,43). Hb Chesapeake was the first mutation described in these genes, and results from a leucine to arginine substitution at position 92 in the α-chain (*HBA* gene). The latter was found in an 81-year-old man and, after a family study, was identified in other fifteen familiar members. The oxygen dissociation curve showed a shift to the left, typical of high oxygen affinity haemoglobins (44).

Currently, there are more than 100 high oxygen affinity variants described for these genes, the most frequent being associated with the *HBB* gene. Transmission in affected patients is usually autosomal dominant (45). Most of the high-affinity variants described thus far have substitutions at one of three regions that are crucial for haemoglobin function: (1) the α1β2 interface; (2) the C-terminal end of the β-chain; and (3) the 2,3-BPG binding site (5). Individuals diagnosed with high affinity Hb tend to be asymptomatic and have high Hct, which is results in hyperviscosity (21).

2.2.2.2 *BPGM* gene

The enzyme biphosphoglycerate mutase (BPGM) is responsible for the conversion of 1,3 bisphosphoglycerate (1,3-BPG) to 2,3 bisphosphoglycerate (2,3-BPG) and regulates the affinity of Hb to oxygen through 2,3-BPG levels. The affinity of Hb to oxygen decreases by the binding of 2,3-BPG, which results in a shift in the oxygen affinity curve to the right, and thus the oxygen is delivered to the tissues (16). Mutations in *BPGM* gene are extremely rare and lead to a decrease of 2,3-BPG levels that consequently causes a shift in the oxygen affinity curve to the left. To balance the decrease of oxygen that is delivered to the cells, there is an increase in the production of RBC, and thus erythrocytosis (13,16). Autosomal and recessive cases have been associated with mutations in *BPGM* gene (46).

3. Diagnosis and Treatment

A correct diagnosis is crucial where there is suspicion of erythrocytosis. For this reason, and as mentioned in section 1.2, acquired causes need to be first excluded (10,47,48). It is also important to screen for conditions like splenomegaly, thrombocytosis and leucocytosis whose presence excludes the possibility of CE (48). The first steps in the screening for CE should be gathering information on the family history and determining serum EPO levels. This information is important for the decision of which molecular tests must be done and in which order. When possible, the determination of P50 and the evaluation of the presence of high oxygen affinity variants of Hb are recommended (10,47,48).

Nowadays, with the introduction of the Next Generation Sequencing (NGS) technique it is possible to perform a more complete and detailed analysis of several genes simultaneously. Moreover, it should be noted that sequencing of the genes associated with erythrocytosis is mandatory for the final diagnosis (5,47,49). The most recent diagnostic algorithm was established by members of MPN&MPNr-Euronet, a network of experts in the molecular diagnosis of myeloproliferative neoplasms and congenital diseases, and is based on the determination of EPO levels and p50 (10,47).

The first line of treatment to erythrocytosis has been phlebotomy, with the purpose of maintaining the value of haematocrit below 0.45. However, low doses of aspirin are also sometimes given in order to decrease the occurrence of possible thrombotic events (10,48).

Aim

The main goals of this project are:

- Identification of mutations associated with CE through molecular studies, in a group of patients with unknown cause of CE, followed in Serviço de Hematologia Clínica do Centro Hospitalar e Universitário de Coimbra (SHC-CHUC), during the last two years;
- Develop skills on molecular techniques as DNA extraction, polymerase chain reaction (PCR) and Sanger sequencing;
- Prediction of the pathogenicity of the mutations found, using *in sílico* tools;
- Update of the online databases and literature.

Material and Methods

1. Analysis of blood samples

The blood samples used in this study were from patients followed in SHC-CHUC or referred from other Portuguese or international hospitals. Studied patients were mainly male and from Portugal (one hundred and eleven samples were from Portugal, six samples were from Turkey, one from Italy and seven from Spain). Molecular studies were performed in the laboratory of the Erythrophatology and Iron Metabolism Unit, SHC-CHUC.

Molecular studies consisted of DNA extraction, amplification of DNA through PCR, agarose gel electrophoresis to confirm the amplification of DNA and Sanger sequencing. Laboratory tests were guided by the clinical and familial history and EPO levels and include Sanger sequencing of the genes: *EPOR* (exons 7 and 8), *SH2B3* (all gene), *VHL* (all gene), *EGLN1* (all gene), *EPAS1* (exon 12), *BPGM* (all gene), *HBB* (all gene) and *HBA* (all gene). New mutations were analysed by *in sílico* analyses to classify its pathogenicity.

➢ DNA Extractions

DNA was extracted from blood samples collected in EDTA, using the following DNA extraction kit: QIAamp® DNA Blood and PureLink™ gDNA Blood. The DNA extractors used were QIAcube 10600® (QIAGEN) and iPrep™ (Invitrogen).

➢ Amplification of DNA by PCR

The main goal of PCR it was obtain a high number of fragment copies of the specific genes to increase the DNA amount. For each gene, different PCR reaction were made and for all PCR reactions were used 2µL of DNA. The thermocycler used in the PCR reactions was the BioRad C1000™ Thermal Cycler and the reagents were from QIAGEN®. PCR reactions and primers for the genes studied are in annex I.

➢ Agarose Gel Electrophoresis

The amplification of DNA by PCR was confirmed in a 2% agarose gel. This gel was prepared with 1g of agarose mixed in 50mL 0.5% TBE (Tris 1.78M; EDTA 0.04M; Boric Acid 1.77M). The solution was heated until the agarose was completely dissolved, and then were added 4µL of SYBR™ Safe. 4µL of PCR samples was load into the gel mixed with a drop of loading dye. Finally, the gel was run at 120V for 25 minutes. At the end of the run, DNA fragments were visualized with UV light and photographed.

- **HPLC**

High-Performance Liquid Chromatography (HPLC) was performed in the Premier Hb9210 resolution for the analysis of Hb variants and quantification of Hb A_2 and Hb F. This analysis was performed through the ion-exchange column that has been equilibrated with respect to pH and ionic the hemoglobin species.

- **Sanger sequencing of the genes**
  - **Purification of the PCR products by EXOSAP®**

The purification of PCR samples it was performed with EXOSAP®. First, it was made a digestion of: primers, Taq polymerase, amongst others compounds of the PCR reaction, except DNA double strand. To that, it was added 0.75µL of EXOSAP® to 3µL of sample. Then, purification it was performed in a thermocycler in to steps, which are the following: 15 minutes at 37°C and 15 minutes to 80°C.

  - **Sequencing and precipitation reaction**

The sequencing reaction of the purified PCR products was performed by BigDye® Terminator v1.1 Cycler Sequencing (Applied Biosystems, Carlsbad, CA, USA) kit. This kit contains a buffer, DNA polymerase, dNTP's and ddNTP's marked with 4 different fluorochromes. The sequencing reactions were prepared with: 6.5µL of dH_2O, 1µL of BigDye, 0.5µL of forward/reverse primer and 2µL of purified sample. Then, the sequencing reaction was performed in the termocycler with the following conditions: 90°C/20s/1 cycle; 60°C/2minutes/24cycles; 4°C/forever. After this, the elimination of BigDye® was made through the application of sequencing sample in a resin column, which was centrifuged 4 minutes and 30 seconds at 3290rpm. The collected product was loaded into a sequencing plate and analysed on the sequencer ABI PRISM 310 (Applied Biosystems®).

- ***In sílico* analysis**

In sílico analysis was performed using five tools that predicts the possible consequences of the amino acid changes on the structure and function of the proteins using straightforward physical and comparative considerations. A mutation is considered pathogenic when 3/5 of *in sílico* tools predict this. The tools used were the following:

- **PolyPhen2-** http://genetics.bwh.harvard.edu/pph2/ (50);
- **Fathmm -** http://fathmm.biocompute.org.uk/ (51);
- **MutationTaster-** http://www.mutationtaster.org/ (52),
- **MutationAssessor-** http://mutationassessor.org/r3/ (53);
- **Provean-** http://provean.jcvi.org/index.php (54).

2. Update of the online databases and literature

The online databases used to do an update of the online databases and literature were accessed in September 2017, and were the following:

- www.erythrocytosis.org (figure 4.1)
- portal.biobase-international.com (figure 4.2)
- www.lovd.nl/3.0/ (figure 4.3)
- exac.broadinstitute.org/ (figure 4.4)
- www.ensembl.org/index.html (figure 4.5)
- globin.bx.psu.edu/hbvar/menu.html (figure 4.6)
- 152.99.75.168/KRGDB (figure 4.7)
- www.ncbi.nlm.nih.gov/pubmed/ (figure 4.8)
- www.ithanet.eu/ (figure 4.9)

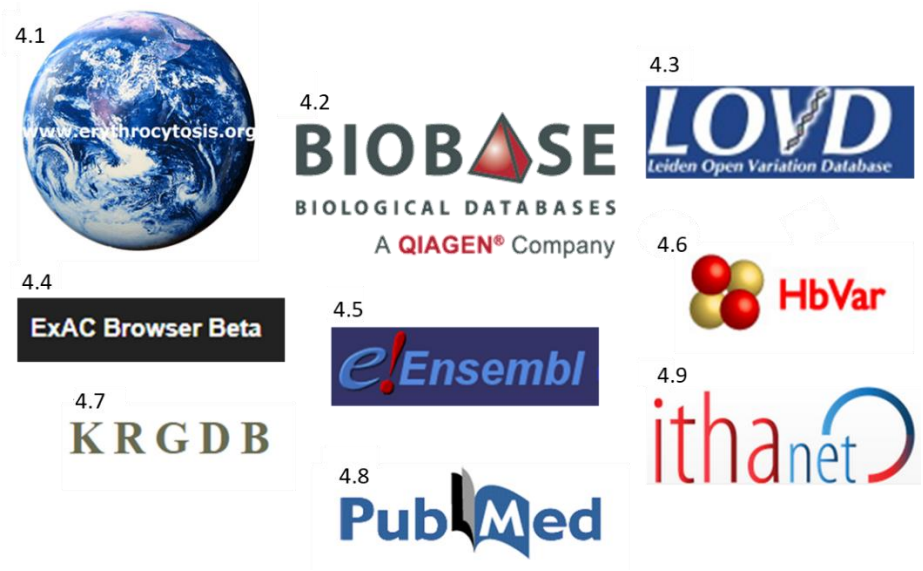

Figure 4- Icons of the online databases.

4.1- Database Erythrocytosis; 4.2- Database Biobase; 4.3- Database LOVD; 4.4- Database ExAC; 4.5- Database Ensembl; 4.6- Database HbVar; 4.7- Database KRGDB; 4.8- Database PubMed; 4.9- Database Ithanet.

Results

1. Analysis of the study of 125 patients with CE

VHL gene mutations

In *VHL* gene 3 different mutations were found (2 missense and 1 nonsense) in 3 different patients. All mutations were found in the heterozygous state. Unfortunately, until now was not possible to perform a familiar study in these patients to understand the inheritance of the mutations with the clinical.

In Patient 1, a 55-year-old male, the change of C>T nucleotide in position 74 (c.74C>T) leads to a Pro>Leu amino acid substitution in codon 25 (p.Pro25Leu) (figure 5), which by *in sílico* analysis was predicted to be benign (table 3).

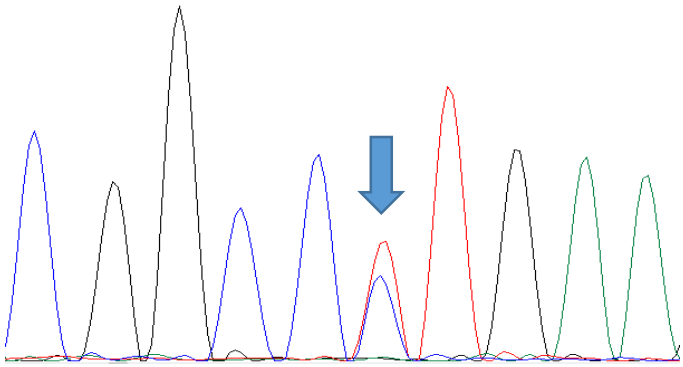

Figure 5- *VHL* gene mutations in Patient 1.

Identification of the *VHL* c.74 C>T mutation, in heterozygous state, in *VHL* gene by Sanger Sequencing.

Table 3- *In sílico* analisys of the *VHL* c.74C>T; p.Pro25Leu mutation.

***In sílico* tools**	**Result**
PolyPhen2	Benign
Fathmm	Damaging
MutationTaster	Polymorphism
MutationAssessor	Functional Impact Neutral
Provean	Neutral

In Patient 2, a 63-year-old male, a change of G>T in position 154 (c.154G>T) results in a stop codon, and then a truncation of the long form (pVHL30) of the protein (p.Glu52*) (figure 6).

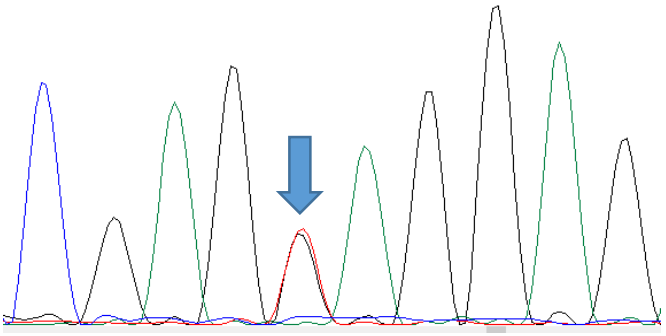

Figure 6- *VHL* gene mutations in Patient 2.

Identification of the *VHL* c.153 G>T mutation, in heterozygous state, in *VHL* gene by Sanger Sequencing.

In Patient 3, a 9-year-old female, the change of C>T nucleotide in position 241 (c.241C>T) results in a Pro>Ser amino acid substitution in codon 81 (p.Pro81Ser) (figure 7), which by *in sílico* analysis was predicted to be damaging (table 4).

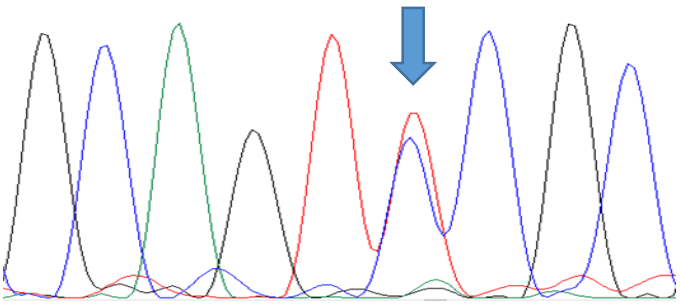

Figure 7- ***VHL* gene mutations in Patient 3.**

Identification of the *VHL* c.241 C>T mutation, in heterozygous state, in *VHL* gene by Sanger Sequencing.

Table 4- *In sílico* analisys of the *VHL* c.241C>T; p.Pro81Ser mutation.

***In sílico* tools**	**Result**
PolyPhen2	Damaging
Fathmm	Damaging
MutationTaster	Polymorphism
MutationAssessor	Functional Impact Low
Provean	Deletirious

EGLN1 gene mutations

In *EGLN1* gene we found 1 missense mutation in heterozygous state, in a male patient with 38 years. A change of G>C in position 1216 (c.1216 G>C) results in a Gly>Arg amino acid substitution in codon 406 (p.Gly406Arg) (Figure8). By *in sílico* analysis it is predicted to be damaging (table 5).

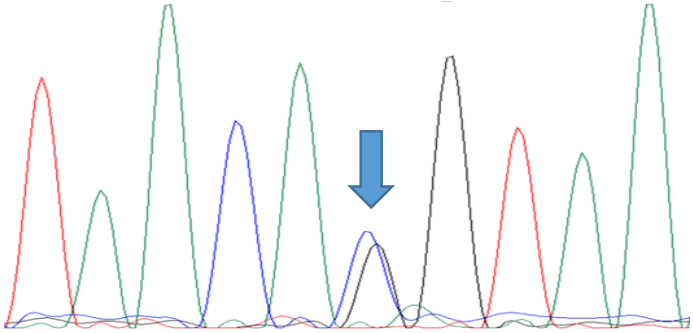

Figure 8- *EGLN1* gene mutations in Patient 4.

Identification of the *EGLN1* c.1216 G>C mutation, in heterozygous state, in *EGLN1* gene by Sanger Sequencing.

Table 5- *In sílico* analisys of the *EGLN1* c.1216 G>C; p.Gly406Arg mutation.

***In sílico* tools**	**Result**
PolyPhen2	Benign
Fathmm	Damaging
MutationTaster	Disease Causing
MutationAssessor	Functional Impact medium
Provean	Damaging

HBB gene mutations

In *HBB* gene we found a G>A change in 328 position (c.328 G>A), in the heterozygous state (figure 9), results in a Val>Met amino acid substitution in codon 110, a known Hb variant with high oxygen affinity called Hb San Diego. A presence of a peak in HPLC confirmed a high oxygen affinity variant (figure 10).

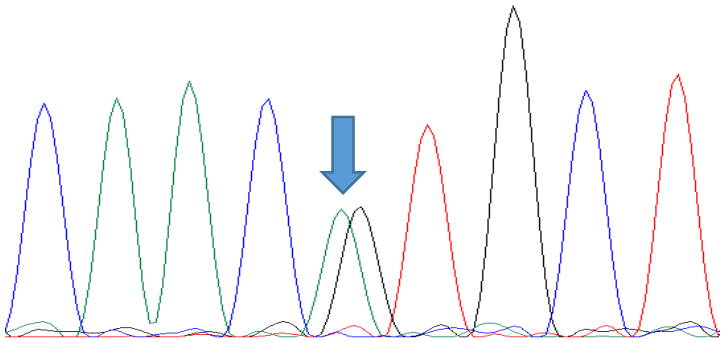

Figure 9- *HBB* gene mutations in Patient 5.

Identification of Hb San Diego in *HBB* gene by Sanger sequencing analysis in patient 5.

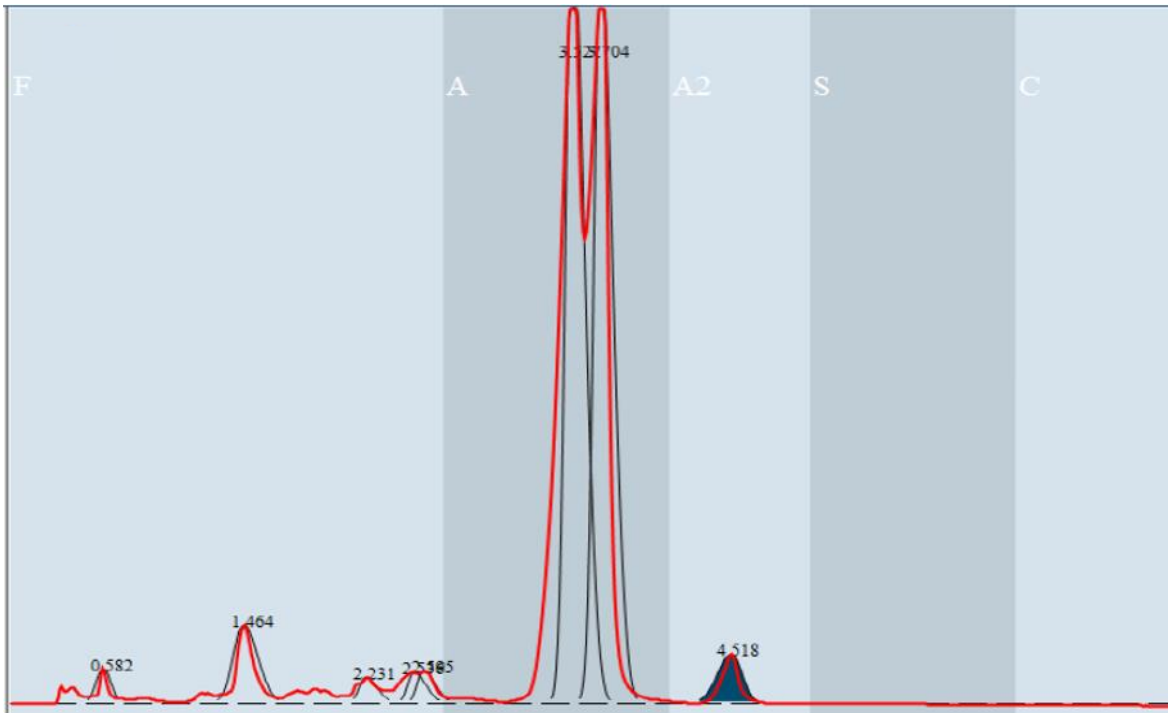

Figura 10- HPLC of patient 5.

HPLC showing the presence of a peak that confirms a high oxygen affinity variant (AXA2 X= 41%).

2. Mutations and new genes associated with Congenital Erythrocytosis – an update of online databases a review of literature

2.1 Update of online databases

CE is a rare disease and consequently the available clinical information on the progression of this disease is sparse. There are around 200 mutations associated with CE. Over 100 of this mutations are responsible for causing high affinity Hb variants (5,19,55). Currently, there are online databases to help establishing the genotype/ phenotype for new mutations or genes founded and where clinical and laboratory data can be recorded and compared (5). This section summarizes the mutations described so far in the genes associated with CE (8 genes to date).

To date, there are 27 mutations (missense/nonsense, small deletions and small insertions) described in *EPOR* gene associated with CE type 1, and the most common mutations are nonsense and located in exon 8. The most recent mutations described are also located in exon 8: p.Cys338Tyr and p.Thr341Met. Functional studies of these mutations demonstrate a gain-of-function in the EpoR signalling cascades of STAT5, promoting an increase in proliferation and differentiation and a decrease in the apoptosis of erythroid progenitors (effect on STAT5 pathways) (56).

Mutations in *SH2B3* gene are associated with myeloproliferative neoplasms and are somatic mutations that are not valued in the presence of CE (57). However, germline mutations result in a decrease of LNK activity, which will not act as a negative regulator in the JAK/STAT pathway. Some authors show that germline mutations in *SH2B3* gene in individuals JAK2-negative and diagnosed with IE could justify this. To date, there are 5 missense mutations described in this gene related to CE type 1 (58). The first mutations found in *SH2B3* gene related to CE was p.Glu208Gln and p.Glu400Lys. The first one is located in the PH domain and leads to premature termination of the protein translation and the second one is located in the functional SH2 domain of LNK (55,58).

To date, there are 20 mutations described (19 missense and 1 nonsense mutations) associated with CE type 2 (*VHL* gene mutations). In addition to Chuvash *VHL* mutations, the mutation p.Met54Ile showed a recent syndrome named “Moroccan erythrocytosis”, because it was found in three individuals originate from northern Morocco. This suggests a single founder to this mutation. All patients are homozygous to this mutation and studies showed a loss-of-function of the VHL protein (25).

There are three isoforms of PHD (PHD1, PHD2, PHD3), but just mutations in PHD2 are associated with CE (5). The p.Trp334* truncation mutation is located in the catalytic domain, a highly conserved region of the protein, and results in a stop codon and consequently in a non-functional protein. The mutation p.Pro304Leu is also located in the catalytic domain (32,35). To date, 30 mutations (missense/nonsense, small deletions and small insertions) in *EGLN1* gene, of which missense mutations are the most common, were described and associated with CE type 3.

There are 12 mutations (11 missense and 1 small deletion mutations) described in *EPAS1* gene related to CE type 4. This gene encodes the transcription factor HIF2α, which is the one isoform that is able to induce the EPO transcription. All mutations described leads to a gain-of function of the HIF2α and are located in exon 12, except p.Tyr532His mutation that is located in exon 9. The carrier patient of this mutation, in addition to having erythrocytosis, also develop pheochromocytoma/paraganglioma (5,13,36,55).

Regarding high oxygen affinity Hb variants there are more than 100 variants described in the *HBA* and *HBB*, all compiled essentially in the online database HbVar (globin.bx.psu.edu). The most recent high affinity Hb variant described is called Hb Seoul [HBB: c.259G>A, p.Ala87Thr] (59). This variant was observed in a Korean man, who had headaches and nausea, high Hb levels and normal EPO levels (63), and can be found in the Korean database KRGDB (152.99.75.168/KRGDB/). Only new cases of high oxygen affinity Hb variants are recorded, thus it is impossible to determine their incidence and prevalence at the population level.

Concerning mutations in *BPGM* gene there are only four cases described with erythrocytosis caused by germline mutations (3 missense mutations and 1 small deletion). The most recent mutation described was in a 27-year-old man with erythrocytosis. This missense mutation, identified by whole-genome sequencing, resulted in the substitution of arginine with histidine at codon 90 (c.269G>A, p.Arg90His) (46). Table 6 summarizes the number of mutations described to date for each gene, as well as the type of mutation. Additionally, all mutations are described in annex II, except for *HBA* and *HBB* genes.

Table 6- Resume of the number of mutations described and the type of mutation for each gene.

GENE	MUTANT PROTEIN	Nº OF MUTATIONS DESCRIBED TO DATE	TYPE OF MUTATION
EPOR	Loss of the negative regulatory domain	25	Missense/ Nonsense Small deletions
SH2B3	Loss of the inhibitory activity of JAK2	5	Missense
VHL	Loss-of-function	20	Missense/ Nonsense
EGLN1	Loss-of-function	30	Missense/ Nonsense Small deletions/ insertions
EPAS1	Gain-of-function	12	Missense Small deletion
HBB/ HBA	Increase Hb affinity for O_2	+100	Missense
BPGM	Reduced synthesis of 2,3- BPG	4	Missense mutations Small deletion

The database erythrocytosis.org aims to register and collect new data of patients with congenital or idiopathic erythrocytosis and patients with polycythaemia Vera with rare somatic JAK2 mutation. In addition, this database aims to characterize these diseases by clinical examination, haematological, biochemical and molecular genetic analyses and collect data about clinical course, treatment, amongst other features. Intertwining databases would contribute to centralize information and hence simplify and accelerate the understanding of the molecular mechanisms of this disease. Mutations described in annex II were updated in the database erythrocytosis.org.

2.2 New genes associated with CE

To date, and as mentioned before, there are eight genes known as associated with CE: *EPOR*, *SH2B3*, *VHL*, *EGLN1*, *EPAS1*, *HBB*, *HBA* and *BPGM*. However, in 70% of patients suspected of having CE the cause is unknown, which reveals that further studies are needed. During the last years, scientists have made an effort to find the "missing" genes causing CE (19,55).

An article published in 2013, described the study of 70 unrelated patients suspected of having CE. All the known casual genes were screened (19). Molecular studies were guided according to the data on serum EPO levels, p50, study of haemoglobins by HPLC and familial history, based on the algorithm publish by McMullin (19,48). As a result, the authors identified a molecular cause in 25/70 patients studied, they found new mutations in *JAK2*, *HBB*, *EPOR*, *VHL* and *EGLN1* genes.

Mutations in *JAK2* gene were somatic mutations, so this allowed the reclassification as polycythaemia Vera in some patients.

Sequencing of the *HBB* and *HBA* genes showed high affinity haemoglobin variants in 14 patients and a new mutation in *EGLN1* was found in a French woman and in three members of her family (19). Two mutations were found in *VHL* gene. The first one was found in homozygous state and was associated with CE and the second one was found in heterozygous state. The latter was inherited from the father, who has normal haematological parameters. Heterozygous *VHL* mutations has been described in the literature, although the mechanism of this is not understand yet. This study reinforces that there must be other genes involved on appearance of erythrocytosis (19).

Another study carried out in 2016, show the importance of the use of NGS panel for the diagnosis of idiopathic erythrocytosis (55). 125 idiopathic erythrocytosis patients were screened, with high suspicious of CE. New genes were included in the NGS panel: *HIF3A*, *EGLN2*, *EGLN3*, *HIF1AN*, *EPO*, *KDM6A*, *GFI1b*, *BHLHE41*, *OS9*, *ZNF197* (55,60).

Fifty one exonic variants were identified in 57 patients, and all variants were validated by Sanger sequencing. Additionally, new variants were found in novel genes, *EPO* and *BHLHE41*, which had not been previously associated with CE. A heterozygous variant (a frameshift) found in *EPO* gene showed a possible association of this gene with CE, because the same variant was found in patient's father, who presented with high Hct and Hb levels. In *BHLHE41* gene, the variant that were found by *in sílico* analysis was classified as benign. Although the *EGLN2*, *HIF3α* and *OS9* genes were associated with the oxygen-sensing pathway, no variants were identified (55). The NGS results reinforce that this technique can be a powerful tool in the genetic investigation of patients with erythrocytosis, since with Sanger sequencing just about 20-30% of the patients are correctly diagnosed (55).

The studies concluded so far have been enlightening and further indicate that the molecular causes of erythrocytosis may be varied and in need of identification. The search for candidate genes is important and can be a solution for many patients with IE. NGS is therefore a powerful tool though the detection of new variants or genes with unknown significance.

Discussion

CE is a rare disease and to date there are 8 genes described and associated with that. Although researchers have been made an effort to find new mutations and genes related to CE, about 70% of the patients with suspicious of CE have unknown cause or are waiting for the diagnosis (5,19,55). Mutations in *EPOR* and *SH2B3* genes are associated with primary CE and are the less common. On the other hand, mutation in genes involved in oxygen-sensing pathway (*VHL*, *EGLN1* and *EPAS1* genes) are related to secondary CE and are the most common. Moreover, mutations in *HBA*, *HBB* and *BPGM* genes lead to high oxygen affinity variants of Hb, also associated with secondary CE (13). In this study we found a molecular cause in 5 of 125 patients studied with suspicious of CE. The mutations found (*VHL*, *EGLN1* and *HBB* genes) are all associated with secondary CE.

***VHL* mutations**

VHL mutations are associated with CE type 2 that is characterized by high levels of Hb, Hct. However, the EPO levels usually are in a normal range. Haematological parameters of patients with *VHL* mutations are in accordance with CE type 2 (table 7). CE type 2 is a recessive disease and there are described cases of individuals heterozygous for *VHL* mutations, but the mechanism of this is not understand so far (55,61). In this study, all *VHL* mutations were found in heterozygous state. So, a familiar study is important to better understand the inheritance of these mutations. However, the family's patients were not able yet.

Patient 1 show a mutation c.74 C>T; p.Pro25Leu, which are located in the exon 1, between the two translational initiation sites (25). This mutation is associated with *VHL* disease, and the majority of disease-causing mutations have been found downstream of the second translational initiation site (+54) and has been associated with a sporadic pheochromocytoma, but in some cases the pathogenicity was not confirmed. So, there are conflicting data about mutations located in the first 53 codons, such as the p.Pro25Leu that could have a pathogenic role. However, this variant is consider a rare variant, which may make the genetic counselling difficult (25,55,62). In fact, *in sílico* analysis predict that this mutation is benign. However, Patient 1 shows high levels of Hb and Hct with normal levels of EPO that are normal in CE type 2 (table 7).

In Patient 2, the mutation c.154 G>T; p.Glu52* leads to a translation termination of the long VHL isoform (pVHL30). However, this mutation does not stop the translation of the short VHL isoform (pVHL19) because of their translational initiation site +54 (55). Despite nonsense mutations in *VHL* gene are more associated with pheochromocytomas or VHL disease than erythrocytosis,

there is a nonsense mutation described (p.Glu10*) related to CE and the p.Glu52* mutation was found in a NGS study of idiopathic erythrocytosis patients (5). But, the role of this mutation regarding erythrocytosis needs to be elucidate, because of its heterozygous state (5,39,55). Patient 3 shown a p.Pro81Ser mutation, predicted to be damaging by in *sílico* analysis. Although this missense mutation was described as confer an increased risk of renal cell carcinoma and haemangioblastomas, was found here in a patient with erythrocytosis (63).

Table 7- Clinical features of the patients with *VHL* gene mutations.

***VHL* GENE**			
	Patient 1	**Patient 2**	**Patient 3**
EPO	7.1	12.4	4.9
HB	17.5	18.3	13.8
HCT	51.8	54.0	40.0
RBC	6.0	5.9	5.8
AGE	55	63	9
GENDER	Male	Male	Female
ORIGEN	Portugal	Portugal	Portugal
FAMILIAR HISTORY	Unknown	Unknown	Unknown
MUTATION	c.74 C>T; p.Pro25Leu rs35460768	c.154 G>T; p.Glu52* rs373068386	c.241C>T; p.Pro81Ser rs104893829

Reference values:
Male: RBC: 4.50-5.50 x 10^6/µL; Hb: 13.0-17.0 g/dL; Hct: 40.0-50.0 %; EPO: 4.3-29.0 mUI/mL
Female: RBC: 4.0-5.2 x 10^6/µL; Hb: 11.5-15.5 g/dL; Hct: 35.0-45.0 %; EPO: 3.0-30.0 mUI/mL

EGLN1 mutations

PHD2 is the major player in the oxygen-sensing pathway, controlling the stability of HIF2α under normoxia/hypoxia conditions (64). Mutations in *EGLN1* are associated with CE type 3 and the haematological parameters are in accordance with this type of erythrocytosis (table 8). These mutations cause a loss-of-function and have an autosomal inheritance (5,35). In fact, Patient 4 had familiar history of phlebotomies by paternal side (table 8). By *in sílico* analysis p.Gly400Arg was predicted to be damaging and was described in a patient with erythrocytosis for the first time in this document. The majority of mutations in *EGLN1* are located in exon 1. However, the p.Gly400Arg is located in exon 4 and, to date, there are just one mutation described (p.Arg398*) in this exon and result in truncated PHD2 (65).

Table 8- Clinical features of the patient with *EGLN1* gene mutations.

***EGLN1* GENE**	
	Patient 4
EPO	10.2
HB	18.3
HCT	53.2
RBC	5.56
AGE	38
GENDER	Male
ORIGEN	Portugal
FAMILIARY HISTORY	Father, paternal aunt and paternal cousin are under phlebotomy
MUTATION	c.1216 G>C; p.Gly406Arg

Reference values:
Male: RBC: 4.50-5.50 x 10^6/µL; Hb: 13.0-17.0 g/dL; Hct: 40.0-50.0 %; EPO: 4.3-29.0 mUI/mL

HBB mutations

Oxygen is transported to the tissues bound haemoglobin in the blood. The oxygenation and deoxygenation of haemoglobin occurs at the heme iron binding site and the affinity for oxygen depends on the haemoglobin. Mutations in *HBB* gene results in a high oxygen affinity haemoglobin variants and have an autosomal dominant inheritance (42,69). Patient 5 showed to have a high oxygen affinity variant suggested by a decreased P50 (table 9), and its presence was confirmed by Sanger sequencing and HPLC, showed an Hb San Diego variant. It was the first time that this variant was observed in a Turkish family. The autosomal inheritance was confirmed by the presence of erythrocytosis in paternal family's members (father and grandmother), who have history of phlebotomy (table 9). Hb San Diego was observed for the first time in a Filipino family, and since them have been described in others families, such as Anglo-Saxon, Japanese , Indian and Caucasian (51,71,72,73).

Table 9- Clinical features of the patients with *HBB* mutations.

***HBB* GENE**	
	Patient 5
EPO MUI/ML	12.4
HB G/DL	16.9
HCT %	50.6
RBC 10^6/µL	5.7
P50	Low
HPLC	AXA2 X=41%
AGE	15
GENDER	Female
ORIGEN	Turkey
FAMILIARY HISTORY	Father (history of phlebotomy), paternal grandmother (history of phlebotomy), paternal aunt and the son of paternal uncle all have erythrocytosis
MUTATION	Hb San Diego: β 109 (G11)Val→Met rs33969677

Reference values:
Female: RBC: 4.0-5.2 x 10^6/µL; Hb: 11.5-15.5 g/dL; Hct: 35.0-45.0 %; EPO: 3.0-30.0 mUI/mL

Update of online databases and a review of literature

Recently, new mutations associated with CE were unveiled/discovered. This new data contributed to diagnose patients with CE phenotype but unknown genotype. However, the number of patients without diagnosis is still high. For instance, this is the case of heterozygous *VHL* mutations, whose molecular mechanism has not yet been explained. With the development of technology, powerful techniques have appeared, such as NGS. This technique allows massive sequencing and improved and accelerated the search for known and new genes associated with CE.

Online databases are important tools to share information among the scientific and medical communities. However, these online databases lack important information. Which delays research and limits diagnosis in patients with CE-like phenotypes. Updating and intertwining the online databases would be of utmost importance.

Conclusion

In this study we identified a CE molecular aetiology in 5/125 patients studied. We found three mutations in *VHL* gene, one mutation not previously described in *EGLN1* gene, and one high oxygen affinity Hb variant (Hb San Diego) in *HBB* gene.

One hundred and twenty patients (96%) remain without a definitive diagnosis, which demonstrates that a further study is need to better understand this disease. The use of NGS technology can be helpful in the study of CE, which will allow to establish a connection with other genes that may be involved in CE and that have not been studied so far. In addition, an update and a intertwining of the online databases can be useful due to the increase and sharing of information between the experts.

Final Notes

My internship started on September 2016 and ended on September 2017. In addition to my project, I had the opportunity to learn new techniques performed in the lab, such as MLPA and NGS, amongst others. Also, I had the opportunity to learn about other genetic diseases related to red blood cells, which allow me to expand my knowledge on other red blood cell diseases besides CE.

During this time I collaborated in several projects and attended meetings, which were the following:

- Biobank project in Paediatric Hospital of CHUC;
- Poster presentation in the Biochemistry Day of University of Aveiro (April 2017) (annex III);
- Poster presentation and oral communication in the 12th MPN&MPNr-EuroNet Meeting 2017, Gothenburg, Sweden (May 2017). I was granted by the University of Nantes to attend this meeting (abstract in annex IV);
- Collaboration in a paper:
  Ebru Yılmaz Keskin, Ali Fettah, Ana Catarina Oliveira, Şule Toprak, **Andreia Lopes,** Celeste Bento (2017). "First Observation of Hemoglobin San Diego, a High Oxygen Affinity Hemoglobin Variant, in Turkey" (annex V);
- Poster presentation in "LIX Congreso Nacional de la SEHH y XXXIII Congreso Nacional de la SETH en Málaga, España" (October 2017) (abstract in annex VI).

Finally, I was selected to do an internship through an Erasmus+ programme. This internship will have place in the University Medical Centre of Utrecht (Netherlands). The main goal is to continue this project, and to find/study novel mutations and genes related to CE.

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Annex

Annex I

PCR reactions for *EPOR*, *VHL* and *EPAS1* genes

***EPOR*, *VHL*, *EPAS1* genes**	
Master Mix	6.25 µL
dH_2O	4.00 µL
Q solution	1.25 µL
Forward primer	0.50 µL
Reverse primer	0.50 µL

Sequence of primers used for *EPOR*, *VHL* and *EPAS1* genes

***EPOR* gene**		
TCCTCTGCCTCCATTGTG	Exon 7/8	Forward*
TACTCAAAGCTGGCAGCAGA		Reverse**
CTCCTGCTCATCTGCTTTGG		Forward**
AGGATGGATTGGGCAGAC		Reverse*

***** used in PCR; ** used in Sanger sequencing

***VHL* gene**		
AGCGCGTTCCATCCTCTAC	Exon 1	Forward */ **
ACGATAGCACGGTCTGAAGC		Reverse *
GAGGTTTCACCACGTTAGCC	Exon 2	Forward */**
TCTCAAAAGCACTTTGGGCT		Reverse *
CCTTGTACTGAGACCCTA	Exon 3	Forward *
CTCAGCTTTTGATGGTACTG		Reverse */**

***** used in PCR; ** used in Sanger sequencing

***EPAS1* gene**		
TCTGCAGGAGCTGAGTTG	Exon 12	Forward
AGAGGCACCCACTAGTAAG		Reverse

***** used in PCR; ** used in Sanger sequencing

PCR reactions for *SH2B3* gene

***SH2B3* gene exon 2**			***SH2B3 gene exon 3-8***	
dH_2O	28.0 µL		**dH_2O**	40.0 µL
Q solution	10.0 µL		**Glucose Buffer**	5.0 µL
Q buffer	5.0 µL		**dNTPs**	1.0 µL
Forward primer	2.0 µL		**Forward primer**	1.5 µL
Reverse primer	2.0 µL		**Reverse primer**	1.5 µL
dNTPs	0.8 µL		**Taq polymerase**	0.4 µL
Taq polymerase	0.2 µL		**-**	**-**

Sequence of primers used for *SH2B3* gene

***SH2B3* exon 2**		
CCAGCACTGGGTGTTATG	Exon 2	Forward */ **
CAGCTGGAAAGCCATCAC		Reverse */ **

***** used in PCR; ** used in Sanger sequencing

***SH2B3* exon 3 to 8**		
CTCAGTGTGAATGGTGTTCA	Exon 3 to 5	Forward */ **
GGGCTACCTTATGTCCTGGG		Reverse**
GTACGCTGGAACCCAGACTC	Exon 6	Forward**
GTCTGCAGCAAGCCTCTACC	Exon 7	Reverse**
TCTGGAAGGAAGGAAAGATCA	Exon 8	Reverse */ **

***** used in PCR; ** used in Sanger sequencing

PCR reactions for *EGLN1* gene

***EGLN1* gene exon 1**			***EGLN1* gene exon 2 to 5**	
dH_2O	28.0 µL		**dH_2O**	28.0 µL
Q solution	2.5 µL		**Glucose Buffer**	10.0 µL
Q buffer	1.25 µL		**dNTPs**	1.25 µL
Forward primer	0.75 µL		**Forward primer**	0.75 µL
Reverse primer	0.75 µL		**Reverse primer**	0.75 µL
DNTPs	0.50 µL		**Taq polymerase**	0.50 µL
Taq polymerase	0.2 µL			

Sequence of primers used for *EGLN1* gene

***EGLN1* gene**		
TAACGGCCCCTATCTCTCT	Exon 1	Forward */ **
GGGAAAAGTAAAGGCCAAGC		Forward **
CGTAAGGAAGACGGACAGAA		Reverse */**
GAAGAATCTAAGTGTAAATCAG	Exon 2	Forward */**
TCTGATAAGACTGTCACAGAC		Reverse *
TTGCAGCTCATCTTCATACTT	Exon 3	Forward *
TATGGTAGGACAGGAGGGAA		Reverse */**
TTAGTCTCCCCTGGTTACTG	Exon 4	Forward */**
TCAGGAAACTCAAGCATAGTC		Reverse *
CAGTGGAATGCAGTAGCAG	Exon 5	Forward *
GAAGATTTTGACTGCTGTGAC		Reverse */**

* used in PCR; ** used in Sanger sequencing

PCR reactions for *BPGM* gene

***BPGM* gene exon 1**			***BPGM* gene exon 2 to 3**	
dH_2O	21.0 µL		**Master Mix**	6.25 µL
Q buffer	2.5 µL		**dH_2O**	4.00 µL
Forward primer	0.5 µL		**Q solution**	1.25 µL
Reverse primer	0.5 µL		**Forward primer**	0.50 µL
DNTPs	0.5 µL		**Reverse primer**	0.50 µL
Taq polymerase	0.2 µL			

Sequence of primers used for *BPGM* gene

***BPGM* gene**		
GCCAACTCCTTACTGGTTCA	Exon 1	Forward */ **
ATGTTGCGAACGTTTACATT		Reverse *
CAGTTGAATATAACTTAGAC	Exon 2	Forward */**
TATACCACTTATTAGAGGTT		Reverse */**
TGATGTAGCACTTGCTGTG	Exon 3	Forward */**
CACTATTCTAATCAGTAGTTCAC		Reverse */**

* used in PCR; ** used in Sanger sequencing

PCR reactions for *HBB* gene

***HBB* gene**	
dH_2O	21.0 µL
Q buffer	2.5 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNTPs	0.5 µL
Taq polymerase	0.2 µL

Sequence of primers used for *HBB* gene

***HBB* gene**		
GAGCCAAGGACAGGTACGG	Exon 1	Forward */ **
GAGGTTCTTTGAGTCCTTTG		Reverse *
AGACTCTTGGGTTTCTGA	Exon 2	Forward */**
TAGAATGGGAAACAGACGAATG		Reverse *
CAATGTATCATGCCTCTTTGCACC	Exon 3	Forward */**
CCATGAAAGAAGGTGAGGCTGC		Reverse *

* used in PCR; ** used in Sanger sequencing

PCR reactions for *HBA* gene

***HBA* gene**	
dH_2O	28.0µL
Q solution	10.0µL
Q buffer	5.0µL
Forward primer	0.8µL
Reverse primer	2.0µL
DNTPs	2.0µL
Taq polymerase	0.2µL

Sequence of primers used for *HBA* gene

***HBA* gene**		
GGTGCACGAGCCGACAGCGC	*HBA1/HBA2*	Forward *
CTGGACTTCGCGGTGGCT	*HBA1*	Reverse *
GCAGGCCTGGCACCTCTCAG	*HBA2*	Reverse *
TCCCCACAGACTCAGAGAGAAC	*HBA1/HBA2* Exon 1	Forward **
ATGTTCCTGTCCTTCCCCAC	*HBA1/HBA2* Exon 2	Forward **
AGTTCCTGGCTTCTGTGAGC	*HBA1/HBA2* Exon 3	Forward **
CGCCCACTCAGACTTTATTCAAAG	*HBA1*	Reverse **
TTATTCAAAGACCAGGAAGGGCCG	*HBA2*	Reverse **

* used in PCR; ** used in Sanger sequencing

Annex II

***EPOR* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.634G>T	p.Gly212Cys	(70)	2014
c.1013G>A	p.Cys338Tyr	(71)	2016
c.1023C>T	p.Thr341Met	(71)	2016
c.1138C>G	p.Pro380Ala	(19)	2013
c.1141_1142delCC	p.Pro381Glnfs*2	(72)	2008
c.1195G>T	p.Glu399*	(73)	2002
c.1234delT	p.Ser412Argfs*41	(74)	2011
c.1235C>A	p.Ser412*	(19)	2013
c.1242_1276del35	p.Ser415Hisfs*18	(19)	2013
c.1249G>T	p.Glu417*	(75)	2010
c.1252_1255delGGAG	p.G418Profs*34	(76)	2004
c.1271_1272delTT	p.Phe424*	(72)	2008
c.1273G>T	p.Glu425*	(14)	2001
c.1278C>G	p.Tyr426*	(77)	1998
c.1281dupT	p.Ile428Tyrfs*17	(78)	1997
c.1282_1289dupATCCTGGA	p.Asp430Glufs*26	(79)	1999
c.1285delC	p.Leu429Trpfs*24	(72)	2008
c.1288dupG	p.Asp430Glyfs*15	(80)	1995
c.1299_1305delCCAGCTC	p.Gln434Cysfs*17	(81)	1997
c.1300C>T	p.Gln434*	(82)	1997
c.1311_1312delTC	p.Pro438Metfs*6	(19)	2013
c.1310G>A	p.Arg437His	(19)	2013
c.1316G>A	p.Trp439*	(83)	1993
c.1317G>A	p.Trp439*	(84)	2007
c.1362C>G	p.Tyr454*	(85)	2016
c.1460A>G	p.Ans487Ser	(86)	1996
c.1462C>T	p.Pro488Ser	(87)	1994

***SH2B3* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.232G>A	p.Glu78Lys	(55)	2016
c.622G>C	p.Glu208Gln	(55)	2016
c.901G>A	p.Glu301Lys	(55)	2016
c.1198G>A	p.Glu400Lys	(88)	2011
c.1244G>C	p.Arg415Pro	(55)	2016

***VHL* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.28G>T	p.Glu10*	(5)	2014
c.162G>C	p.Met54Ile	(25)	2015
c.235C>T	p.Arg79Cys	(89)	2005
c.241C>G	p.Pro81Ala	(5)	2014
c.311G>T	p.Gly104Val	(90)	2005
c.370A>G	p.Thr124Ala	(91)	2013
c.376G>A	p.Asp126Asn	(92)	2011
c.376G>T	p.Asp126Asn	(93)	2003
c.388G>C	p.Val130Leu	(93)	2003
c.413C>T	p.Pro138Leu	(94)	2013
c.430G>A	p.Gly144Arg	(95)	2005
c.449A>G	p.Asn150Ser	(96)	2015
c.524A>G	p.Tyr175Cys	(89)	2005
c.548C>T	p.Ser183Leu	(92)	2011
c.562C>G	p.Leu188Val	(93)	2003
c.571C>G	p.His191Asp	(93)	2003
c.574C>A	p.Pro192Thr	(5)	2014
c.574C>T	p.Pro192Ser	(93)	2003
c.586A>G	p.Lys196Glu	(19)	2013
c.598C>T	p.Arg200Trp	(97)	2002

***EPAS1* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.1121T>A	p.Phe374Tyr	(36)	2012
c.1594T>C	p.Tyr532His	(55)	2016
c.1597A>G	p.Ile533Val	(98)	2013
c.1601C>T	p.Pro534Leu	(99)	2009
c.1603A>G	p.Met535Val	(100)	2012
c.1604T>C	p.Met535Thr	(38)	2012
c.1605G>A	p.Met535Ile	(101)	2008
c.1609G>A	p.Gly537Arg	(38)	2008
c.1609G>T	p.Gly537Trp	(37)	2008
c.1617C>G	p.Asp539Glu	(102)	2010
c.1620C>G	p.Phe540Leu	(100)	2012
c.49_51delGAG	p.del17Glu	(55)	2016

***BPGM* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.185G>A	p.Arg62Gln	(16)	2004
c.268C>T	p.Arg90Cys	(103)	1992
c.268G>A	p.Arg90His	(46)	2014
c.61delC	p.Arg21Valfs*28	(103)	1992

***EGLN1* mutations**			
Nucleotide exchanged	**Protein effect**	**References**	**Year**
c.12C>A	p.Asp4Glu	(104)	2013
c.380G>C	p.Cys127Ser	(105)	2014
c.413T>C	p.Val138Ala	(70)	2014
c.471G>C	p.Gln157His	(106)	2011
c.493G>T	p.Pro165Ser	(70)	2014
c.494delC	p.Pro165fs	(107)	2016
c.599C>A	p.Pro200Gln	(65)	2012
c.606delG	p.Met202Ilefs*72	(72)	2008
c.609C>G	p.Asn203Lys	(108)	2012
c.610G>G	p.Lys204Glu	(5)	2014
c.661C>T	p.Gln221*	(32)	2016
c.678dupG	Arg227Alafs*20	(32)	2016
c.715C>T	p.Gln239*	(32)	2016
c.760G>C	p.Asp254His	(65)	2012
c.835_848del14	p.Leu279Thrfs43*	(70)	2014
c.836T>C	p.Leu279Pro	(55)	2016
c.840dupA	p.Arg281Thrfs*4	(72)	2008
c.853G>C	p.Gly285Arg	(5)	2014
c.872A>T	p.Lys291Ile	(108)	2012
c.911C>T	p.Pro304Leu	(32)	2016
c.950C>G	p.Pro317Arg	(34)	2006
c.1000T>C	p.Trp334Arg	(19)	2013
c.1001G>A	p.Trp334*	(32)	2016
c.1010dupA	p.Val338Glyfs*18	(5)	2014
c.1111C>T	p.Arg371Cys	(32)	2016
c.1112G>A	p.Arg371His	(65)	2007
c.1121A>G	p.His374Arg	(31)	2008
c.1129C>T	p.Gln377*	(72)	2008
c.1192C>T	p.Arg398*	(65)	2012
c.1267A>G	p.Lys423Glu	(108)	2012

Annex III

Bases Moleculares da Eritrocitose Congénita

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Introdução

A Eritrocitose Congénita (EC) é uma doença rara que tem como base mutações germinativas nos genes: *EPOR*, *SH2B3* (LNK), *VHL*, *EGLN1* (PHD2), *EPAS1* (HIF2a), *HBB*, *HBA* e *BPGM* [1,2].

Fenotipicamente é caracterizada por um aumento do número de glóbulos vermelhos, um hematócrito elevado e níveis aumentados de hemoglobina (Hb), quando comparados com os valores de referência, de acordo com o género e idade. Nos adultos Hb> 18.5g/dl ou Hct> 52% nos homens e Hb> 16.5g/dl ou Hct> 48% nas mulheres [2].

O diagnóstico é elaborado com base no algoritmo proposto por McMullin *et al.*, 2005. Este algoritmo é baseado na determinação dos níveis séricos de eritropoietina (EPO), que são a ferramenta principal na escolha dos testes moleculares a realizar[4].

Mecanismos Moleculares

➢ EC Primária

A EC Primária é observada quando os níveis de eritropoietina (EPO) são baixos, devido a defeitos intrínsecos dos progenitores eritroides. A ligação de EPO ao recetor EPOR induz a autofosforilação de JAK2, responsável pela ativação de fatores de transcrição, e que por sua vez induz a fosforilação do domínio citoplasmático do recetor EPOR. Após este processo, a proteína STAT5 é homodimerizada, migrando para o núcleo, onde se liga ao promotor do gene a transcrever. No final do processo, a proteína STAT5 sofre desfosforilação e é transportada novamente para o citoplasma. Por outro lado, a proteína LNK tem como função a inibição da atividade da enzima JAK2 [1,2,3].

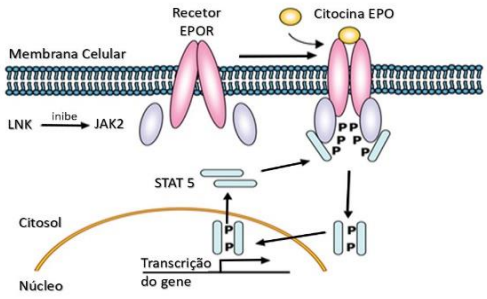

Figura 1- Mecanismo de ligação de EPO ao recetor EPOR. Imagem adaptada [3].

➢ EC Secundária

A EC secundária é o resultado da resposta fisiológica a um estado de hipóxia, resultando no aumento da produção de EPO. Em normóxia a enzima PHD2 hidroxila o fator HIF-2α, que por sua vez se liga a pVHL formando um complexo, que é posteriormente degradado no proteossoma. Em condições de hipóxia existe a ligação da subunidade β à subunidade α do fator HIF. Esta ligação impede a degradação de HIF-2α, mas favorece a sua translocação para o núcleo, que por sua vez conduz à transcrição de EPO. Como consequência, o aumento de EPO leva ao aumento da produção do número de glóbulos vermelhos [1,2,4].

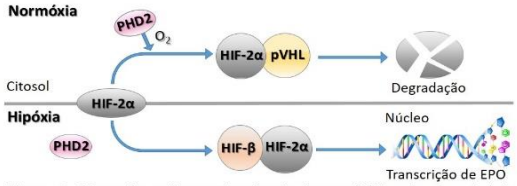

Figura 2- Mecanismo ilustrativo da via de sensibilização ao oxigénio em normóxia e hipóxia. Imagem adaptada [2].

Quando não é possível identificar a causa, a eritrocitose é classificada como **idiopática.** Na tabela 1 está sumarizado a classificação da EC, assim como a patologia e os gene associados a cada uma.

Tabela1- Divisão da Eritrocitose Congénita em primária e secundária e respetivas patologias associadas a cada classificação, bem como as mutações nos genes associados [1,2].

Eritrocitose Congénita	
Primária (↓níveis de EPO)	**Secundária (↑ níveis de EPO)**
Proteínas da via de sinalização EPO/EPOR (devido a mutações nos genes *EPOR* e *SH2B3)*	Hb com alta afinidade para O_2 (mutações nos genes *HBA* e *HBB*)
	Deficiência em 2,3-BPG (mutações no gene *BPGM*)
	Proteínas da via dos sensores de hipóxia (mutações em *VHL*, *EPAS1* e *EGLN1*)
Idiopática- causa desconhecida	

Conclusão

A EC tem como base mutações germinativas em vários genes envolvidos em diferentes mecanismos, nomeadamente a ligação de EPO ao recetor EPOR ou no mecanismo de resposta à hipóxia. Consoante os níveis séricos de EPO, a EC é classificada como primária ou secundária, facilitando assim a escolha dos testes moleculares a realizar.

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Annex IV

Congenital Erythrocytosis: identification of novel mutations in *EGLN1* (PHD2), *SH2B3* (LNK) and *VHL* genes

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Congenital Erythrocytosis (CE) may either be primary or secondary to elevated Epo concentrations. The only known form of primary CE is caused by mutations in the *EPOR* gene. Secondary CE can be caused by congenital defects such as Hbs with increased oxygen affinity, due to mutations in the *HBB*, *HBA* or *BPGM*, or from defects in the components of the oxygen-sensing pathway, namely mutations in *EGLN1*, *EPAS1* and *VHL*. Despite important discoveries in the molecular pathogenesis of CE, in about 70% of the patients the genetic causes remain to be identified.

We studied 54 subjects with CE, followed in our hospital or referred from other hospitals, during the last 2 years. Laboratory testing was guided by the clinical history and EPO levels and included: *HBB*, *HBA*, *BPGM*, *EPOR*, *SH2B3*, *VHL*, *EGLN1* and *EPAS1* Sanger sequencing.

In this study we identified: 2 mutations in *EGLN1*, p.Phe366Leu results in a substitution in a highly conserved region of the PHD2 and was found in a father and son (64 and 39 yo), and Arg227Alafs*20 that was observed in a 34 yo male; 3 mutations in *SH2B3*: p.Arg80Cys, present in phenylalanine zipper which is responsible to mediate the dimerization of LNK, p.Pro308* that belongs to PH domain; and p.Glu400Lys already described. *SH2B3* mutations were found in a 67 yo female, 18 yo male and 85 yo female respectively; 1 mutation in *VHL*, observed in a 55 yo male and results in a p.Pro25Leu substitution. All mutations were found in heterozygous state.

We were able to identify the CE molecular aetiology in 6/54 subjects studied. We found 1 mutation already described and 5 new mutations, which by *in silico* analysis were predicted to be damaging. However, 48 patients still remain without definitive diagnostic. This demonstrates that further study is needed to better understand this disease. The use of NGS technology can be helpful in the study of CE, which will allow establishing a connection with other genes that may be involved in CE and that have not been studied so far.

Annex VI

First Observation of Hemoglobin San Diego, a High Oxygen Affinity Hemoglobin Variant, in Turkey

Türkiye'de Gözlenen İlk Hemoglobin San Diego (Oksijene İlgisi Yüksek Bir Hemoglobin Varyantı) Olgusu

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To the Editor,

Congenital erythrocytosis (CE) or congenital polycythemia represents a rare clinical entity. High oxygen affinity hemoglobin (Hb) variants are a very rare cause of secondary CE. In 1966, Charache et al. [1] published the first case of a Hb variant associated with erythrocytosis, Hb Chesapeake. Since then, more than 220 variants with high oxygen affinity have been discovered and the autosomal dominant inheritance has been confirmed [2].

Many Hb variants have been reported so far from Turkey [3,4,5]. We report herein the first observation of Hb San Diego, a high oxygen affinity Hb variant, from Turkey in a case of CE.

Case: A 15-year-old female patient residing in Kastamonu, Turkey, and examined due to the complaints of occasional headache, fatigue, dizziness, nausea, and chest pain was found to have an elevated Hb level. Erythrocytosis was also present in other family members, including her father and paternal grandmother (Figure 1). Both the father and grandmother had a history of several phlebotomies.

Laboratory data are presented in Table 1. Serum biochemistry, abdominal ultrasonography, and echocardiographic examinations were all unremarkable. In addition to her family history consistent with a disorder transmitted autosomal dominantly, the finding of reduced P50 suggested the presence of a high oxygen affinity Hb. Hb electrophoresis performed with the high-performance liquid chromatography (HPLC) method with the device ZIVAK using the Hb Variant Whole Blood HPLC Analysis Kit yielded no abnormal Hb variant. The examination was repeated with Trinity Biotech's Premier Hb9210 resolution method and displayed the presence of a Hb variant in both the patient and her father (Figure 1). Sanger sequencing analysis confirmed the associated mutation in the β-globin gene [Hb San Diego; β109(G11)Val→Met] (Figure 2).

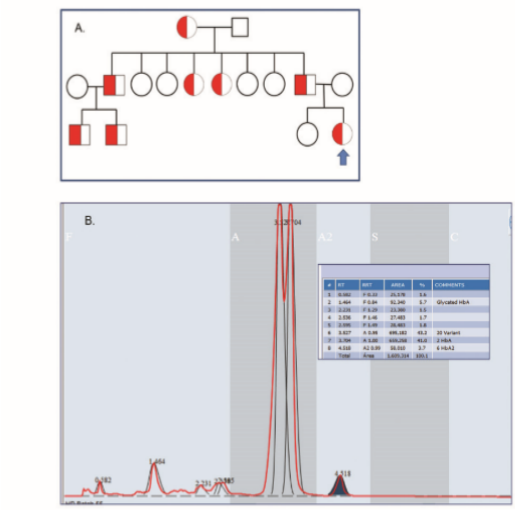

Figure 1. A) Pedigree of the family with erythrocytosis and hemoglobin (Hb) San Diego, illustrating dominant mode of inheritance of erythrocytosis. The propositus is indicated with an arrow; B) high-performance liquid chromatography (premier Hb9210 resolution) showing the presence of Hb San Diego.

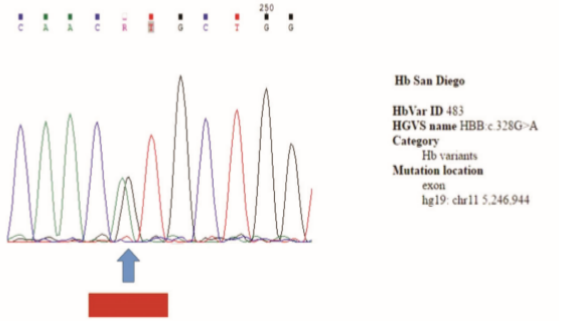

Figure 2. Identification of hemoglobin San Diego in β-globin gene by Sanger sequencing analysis in the index case.

HbVar: Hemoglobin variant.

Table 1. Laboratory findings of the patient at the time of admission.	
Hemoglobin (g/L)	169 (NR: 120-160)
Hematocrit (%)	50.6 (NR: 36-46)
Red blood cells (10^6/μL)	5.72 (NR: 4.1-5.1)
Reticulocyte ratio (%)	1.2 (NR: 0.3-1.5)
MCV (fL)	88.5 (NR: 70.6-95.6)
White blood cells (10^3/μL)	4.4 (NR: 4.5-13.0)
Platelets (10^3/μL)	258 (NR: 150-400)
Ferritin (ng/mL)	12.4 (NR: 12-150)
Vitamin B12 (pg/mL)	421 (NR: 200-820)
Folic acid (ng/mL)	6.3 (NR: 3.0-7.2)
Homocysteine (μmol/L)	5.8 (NR: 5.0-13.0)
Erythropoietin (mIU/mL)	12.4 (NR: 4.3-20.0)
P50* (mmHg)	15.5 (NR: 22.6-29.4)
*P50 is the oxygen tension at which the hemoglobin molecule is one-half saturated. NR: Normal range, MCV: mean corpuscular volume.	

Erythrocytosis may be the clinical manifestation of the presence of a high oxygen affinity Hb. Hb San Diego was first reported in 1974 in a Filipino family [6]. Thereafter, it has been described in subjects of different origins [7,8,9,10,11,12]. Our case represents the first one of Hb San Diego in Turkey. Although Hb San Diego was described as electrophoretically silent [6], it could be clearly identified using the new Trinity Biotech Premier Hb9210 resolution technology.

In their study evaluating 70 patients with CE, Bento et al. [11] sequenced all the genes described as associated with CE and erythrocytosis molecular etiology was identified in only 25 (36%) subjects, a high oxygen affinity Hb being the cause in 14 (56%) of these 25 subjects. Determination of the P50 value, calculated easily from fresh venous blood gas samples, is a practical and useful test, and a decreased value may direct clinicians to order examinations regarding a Hb variant [12]. Some high oxygen affinity Hbs are electrophoretically silent but their identification can be rapidly done by direct sequencing of the globin genes (HBB and HBA).

Management of CE caused by a high oxygen affinity Hb should be personalized, and it should primarily depend on smoking cessation and physical activity. Phlebotomy and platelet aggregation inhibitors' prescription should be evaluated carefully, and blood donation is not advised [2].

Keywords: Abnormal hemoglobins, Hemoglobin San Diego, Hemoglobin variant

Anahtar Sözcükler: Anormal hemoglobinler, Hemoglobin San Diego, Hemoglobin varyantı

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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Annex VI

Eritrocitosis Congénita: Identificación de mutaciones en los genes *EGLN1* (PHD2), *VHL* y *HBB* en un estudio de 50 pacientes

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Introducción: La Eritrocitosis Congénita (EC) puede ser primaria o secundaria a elevadas concentraciones de eritropoyetina (Epo). La única forma conocida de EC primaria es causada por mutaciones en el gene *EPOR* y recientemente se ha asociado el gen *SH2B3*. La EC secundaria puede ser causada por defectos congénitos como Hbs con mayor afinidad por el oxígeno, debido a mutaciones en *HBB*, *HBA* o *BPGM*, o por defectos en los componentes de la vía de detección de oxígeno, a saber mutaciones en *EGLN1*, *EPAS1* y *VHL*. A pesar de que ya se han identificado las causas que están en la base de la EC, cerca del 70% de los individuos aún permanecen sin diagnóstico.

Métodos: Se estudiaron 50 sujetos con EC, seguidos en nuestro hospital o remitidos de otros hospitales, durante los últimos 2 años. Las pruebas de laboratorio se basaron en la historia clínica y los niveles de Epo e incluyeron secuenciación de Sanger de los genes: *HBB*, *HBA*, *BPGM*, *EPOR*, *SH2B3*, *VHL*, *EGLN1* y *EPAS1*.

Resultados: En este estudio hemos identificado: 2 mutaciones en *EGLN1*, p.Phe366Leu que causa una sustitución en una región altamente conservada de la PHD2 y se encontró en un padre y su hijo (64 y 39 años), y la p.Arg227Alafs * 20 que se observó en un hombre de 34 años. En *VHL* se encontraron 2 mutaciones: p.Pro25Leu, en heterozigotia, en un hombre de 55 años. Esta mutación que se clasifica como una variante no patogénica. La segunda mutación encontrada fue p.Glu52 *, que a pesar de ya estar descrita, su papel aún no está claro cuando asociada a eritrocitosis y en heterozigotia. Esta mutación se observó en un hombre de 63 años. Por fin, en *HBB* identificamos una variante de Hb- Hb de San Francisco- en una niña de 16 años.

Conclusiones: En nuestro estudio identificamos la probable causa genética de la EC en 5/50 pacientes. Mientras, 45 pacientes permanecen sin diagnóstico, lo que demuestra que se necesitan más estudios para comprender mejor esta enfermedad. El uso de la tecnología *NGS* puede ser útil en el estudio de EC, lo que permitirá establecer una conexión con otros genes que pueden estar implicados en EC y que no han sido estudiados hasta el momento.